

Use of Sulfated Linked Cyclitols as Heparan Sulfate Mimetics to Probe the Heparin/Heparan Sulfate Binding Specificity of Proteins*

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Heparin and heparan sulfate (HS) are structurally diverse glycosaminoglycans (GAG) that are known to interact, via unique structural motifs, with a wide range of functionally distinct proteins and modulate their biological activity. To define the GAG motifs that interact with proteins, we assessed the ability of 15 totally synthetic HS mimetics to interact with 10 functionally diverse proteins that bind heparin/HS. The HS mimetics consisted of cyclitol-based pseudo-sugars coupled by linkers of variable chain length, flexibility, orientation, and hydrophobicity, with variations in sulfation also being introduced into some molecules. Three of the proteins tested, namely hepatocyte growth factor, eotaxin, and elastase, failed to interact with any of the sulfated linked cyclitols. In contrast, each of the remaining seven proteins tested exhibited a unique reactivity pattern with the panel of HS mimetics, with tetrameric cyclitols linked by different length alkyl chains being particularly informative. Thus, compounds with short alkyl spacers (2–3 carbon atoms) effectively blocked the interaction of fibroblast growth factor-1 (FGF-1) and lipoprotein lipase with heparin/HS, whereas longer chain spacers (7–10 carbon atoms) were required for optimal inhibition of FGF-2 and vascular endothelial growth factor binding. This effect was most pronounced with the chemokine, interleukin-8, where alkyl-linked tetrameric cyclitols were essentially inactive unless a spacer of >7 carbon atoms was used. The heparin-inhibitable enzymes heparanase and cathepsin G also displayed characteristic inhibition patterns, cathepsin G interacting promiscuously with most of the sulfated cyclitols but heparanase activity being inhibited most effectively by HS mimetics that structurally resemble a sulfated pentasaccharide. These data indicate that a simple panel of HS mimetics can be used to probe the HS binding specificity of proteins, with the position of anionic groups in the HS mimetics being critical.

Heparan sulfate (HS)¹ is a glycosaminoglycan that is ubiquitously expressed as a proteoglycan on cell surfaces and

throughout the extracellular matrix (ECM) in most multicellular animals (1–5). The polysaccharide is composed of alternating glucuronic acid and *N*-acetylglucosamine units, which, during biosynthesis, are subjected to a range of modifications such as *O*-sulfation at various positions, *N*-deacetylation, and *N*-sulfation of *N*-acetylglucosamine residues as well as C-5 epimerization of glucuronic acid to iduronic acid. Theoretically up to 48 different disaccharides could occur in HS due to these modifications, although so far only 23 have been identified (2). Nevertheless, as a result of the presence of these different disaccharides, HS chains exhibit remarkable structural heterogeneity. Such diversity is further enhanced by the considerable variation in chain length of the glycosaminoglycan. Heparin, a glycosaminoglycan with a much more restricted cellular distribution than HS, is structurally closely related to HS but has ~80% of its disaccharide units epimerized or sulfated compared with only ~10% of disaccharides being modified in HS (6). The modified disaccharides in HS, however, are concentrated in “hot spots” along the molecular backbone and separated by flexible spacers of low sulfation, rather than being evenly distributed throughout the polysaccharide chain (2, 3).

Both heparin and HS are known to interact with a wide range of functionally diverse proteins, such as growth factors, cytokines, chemokines, proteases, lipases, and cell adhesion molecules (2–4, 6–8). Furthermore, there is increasing evidence to suggest that these proteins bind to unique structural motifs on the HS chains, with different tissues and cell types controlling their ability to interact with various HS-binding proteins by varying their repertoire of sugar sequences. In fact, genetic studies in *Drosophila* and mice (3, 9–11) have demonstrated unequivocally that a lack of specific HS modifications can impair development, whereas mice deficient in HS die at the gastrula stage (12). At the physiological level, it appears that HS can regulate the function of HS-binding proteins in a number of ways, with some well characterized examples being listed below. First, depots of growth factors and cytokines can exist in the ECM bound to HS (13, 14). These can be liberated by ECM-degrading enzymes, such as heparanase, and be made available for important physiological processes such as wound healing and angiogenesis (15, 16). Second, cell surface HS can interact with both HS-binding growth factors and growth factor receptors and, thereby, greatly facilitate signal transduction via such receptors (3, 11, 17, 18). Third, chemokines and cytokines can form stable gradients within tissues by associating with HS chains in the ECM, an interaction that aids leukocyte entry into inflammatory sites (19). Finally, the activity of a

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¹ The abbreviations used are: HS, heparan sulfate; ECM, extracellular

lar matrix; PI-88, phosphomannopentaose sulfate; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; IL-8, interleukin-8; LpL, lipoprotein lipase.

range of enzymes can be modulated by heparin and HS, with the classic example being the ability of heparin to potentiate antithrombin III-mediated inhibition of thrombin (2, 6). Similarly, heparin, and to a lesser extent HS, can both directly and indirectly inhibit the proteolytic activity of neutrophil elastase and cathepsin G (20–22).

Because data are emerging that suggest different HS-binding proteins tend to interact with unique structural motifs within HS, the possibility now exists for the design and synthesis of HS mimetics, which selectively block undesirable protein-HS interactions (23). In fact, we have recently developed an HS-mimetic, called PI-88, that exhibits anti-angiogenic, anti-metastatic, and anti-restenotic activities and that is currently undergoing Phase II clinical trials in cancer patients. This drug, which is composed primarily of sulfated phosphomannopentaose and phosphomannotetraose oligosaccharide units, is believed to exert its biological effects by blocking the enzymatic activity of the ECM-degrading enzyme, heparanase, and by interfering with the action of HS-binding growth factors such as fibroblast growth factor-1 (FGF-1), FGF-2, and vascular endothelial growth factor (VEGF) (24–26). PI-88, however, interferes with a relatively broad range of protein-HS interactions, because it represents a modified natural product containing a mixture of differentially sulfated oligosaccharides. To obtain homogeneous and structurally well defined HS mimetics, a family of totally synthetic molecules, namely sulfated linked cyclitols, was prepared. This study compares the ability of 15 different sulfated linked cyclitols to interact with 10 functionally diverse proteins. It was found that each protein examined exhibited a unique reactivity pattern with the panel of sulfated cyclitols, confirming the view that many HS-binding proteins preferentially bind to unique structural motifs in HS. This study also highlights the selective therapeutic potential of HS mimetics and demonstrates that a relatively simple panel of mimetics can provide considerable information regarding the HS binding specificity of proteins.

EXPERIMENTAL PROCEDURES

Reagents—PI-88, provided by Progen Industries Ltd. (Brisbane, Australia), was prepared as described previously (24, 27) by the sulfation of phosphomanno-oligosaccharides derived from the phosphomannan produced by the yeast *Pichia holsti*. It consists predominantly of sulfated phosphomannopentaose and phosphomannotetraose with its components having molecular masses in the range ~1400 to ~3100 Da. Bovine lung heparin (mean molecular mass ~12.5 kDa) was purchased from Sigma-Aldrich. Porcine mucosal HS (ORG 553) was a generous gift from Organon Int. Bv (Oss, The Netherlands). FGF-1, FGF-2, VEGF₁₆₅, interleukin-8 (IL-8), and eotaxin were supplied by R & D Systems, Minneapolis, MA. Hepatocyte growth factor (HGF) was a generous gift of Dr. Oyvind Hjertner, Institute of Cancer Research and Molecular Biology, Medisinsk Teknisk Senter, Trondheim, Norway. Human platelet heparanase was purified according to the method of Freeman and Parish (28). Bovine lipoprotein lipase (LpL), human leukocyte elastase, and human leukocyte cathepsin G were purchased from Sigma-Aldrich.

Surface Plasmon Resonance—Binding studies were performed, as previously reported (25), on a BIAcore 2000 surface plasmon resonance-based biosensor (BIAcore, Uppsala, Sweden) using either biotinylated bovine lung heparin or biotinylated porcine mucosal HS immobilized on a CM5 sensor chip (BIAcore). Briefly, the carboxymethylated surface of the sensor chip was first activated with a mixture of *N*-hydroxysuccinimide and *N*-ethyl-*N'*-(3-diethylaminopropyl)carbodiimide, NeutrAvidin was then covalently attached to the chip surface, and any residual activated ester groups were blocked by ethanolamine hydrochloride. Biotinylated heparin or biotinylated HS was then bound to the immobilized NeutrAvidin, with the flow cells finally being washed with 4 M NaCl. The different heparin/HS-binding proteins (25–68 nM) were injected into the flow cells of the biosensor, with binding and dissociation each being monitored for 3–10 min. Data points were collected continuously during the binding and dissociation phases, and analysis was based on the response once saturation binding had been reached. Between each cycle, the sensor surface was regenerated with 4 M NaCl. Simultaneous measurements were obtained from the four flow cells

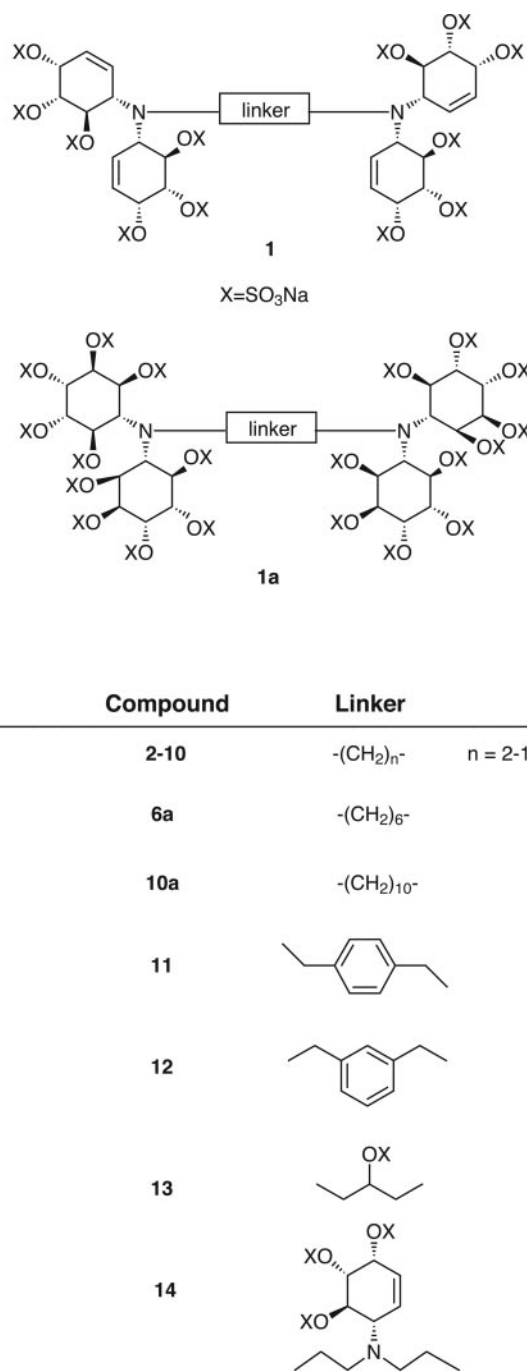


FIG. 1. Structures of the various sulfated linked cyclitols used in this study. All compounds shown are related to 1, with the exceptions being 6a and 10a that are compound 1a derivatives.

with flow cells 1 and 3 containing NeutrAvidin alone (to establish background binding), flow cell 2 containing immobilized biotinylated heparin, and flow cell 4 containing immobilized biotinylated HS. Flow cells 1 and 3 were used as blank reference cells, with the background binding to NeutrAvidin in flow cell 1 and 3 being subtracted from the responses in flow cells 2 and 4. The sensorgrams were analyzed with the aid of the BIAevaluation software version 3.0.2 (BIAcore). Baselines were adjusted to zero for all curves and background sensorgrams subtracted from experimental sensorgrams to produce curves of specific binding.

Enzyme Assays—Heparanase activity was assayed as described previously (29). Cathepsin G activity was determined in 96-well U-bottomed plastic microplates by incubating human leukocyte cathepsin G (40 μl/well, 100 ng/well of enzyme), in the presence or absence of inhibitors, with the fluorogenic substrate *N*-succinyl-Ala-Ala-Pro-Phe-amidomethylcoumarin (Sigma-Aldrich) in 50 mM sodium acetate buffer,

pH 6.6, containing 50 mM NaCl and 0.1% (v:v) Brij 35. The mixture was incubated at 37 °C for 60 min, the reaction was stopped by the addition of 40 μ l/well of 250 mM acetic acid, and fluorescence was determined using a Cytofluor II fluorescence plate reader (PerSeptive Biosystems, Framingham, MA) employing an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Because plastic microplates inhibited elastase enzymic activity, elastase assays were performed in 0.5-ml polycarbonate tubes with reaction mixtures, upon reaction completion,

TABLE I
General properties of heparin/HS-interacting proteins
used in this study

Protein class	Protein	IC ₅₀ ^a	
		Heparin ^b	PI-88 ^c
<i>nM</i>			
Heparin/HS binding growth factors	FGF-1	5	10
	FGF-2	5	34
	VEGF	20	25
	HGF	5	30
Heparin/HS binding chemokines	IL-8	8	>2000
	Eotaxin	25	>2000
Heparin/HS binding enzymes	LpL	2	30
Heparin inhibitable enzymes	Heparanase	160	1260
	Cathepsin G	25	140
	Elastase	2	5000

^a Concentration of heparin or PI-88 required to inhibit protein binding or enzymic activity by 50%. With heparin/HS-binding proteins heparin and PI-88 were assessed for their ability to inhibit the binding of 25 nM FGF-1, 34 nM FGF-2, 25 nM VEGF, 20 nM HGF, 68 nM IL-8, 25 nM eotaxin, or 50 nM LpL to immobilized heparin using an optical biosensor. Enzyme assays were performed as described under "Experimental Procedures."

^b Bovine lung heparin (12.5 kDa).

^c Phosphomannopentaose sulfate.

being transferred to 96-well plastic microplates for reading by the fluorescence plate reader. Human leukocyte elastase (40 μ l/tube, 20 ng/tube of enzyme) was incubated, in the presence or absence of inhibitors, with the fluorogenic substrate MeOSuc-Ala-Ala-Pro-Val-amido-methylcoumarin (ICN Biomedicals, Irvine, CA) in 50 mM Tris-HCl buffer, pH 7.5, containing 50 mM NaCl and 0.1% (v:v) Brij 35. The mixture was incubated at 37 °C for 20 min, the reaction was then stopped by the addition of 40 μ l/well of 250 mM acetic acid, and the mixture was transferred to a 96-well microplate, with fluorescence being determined as for cathepsin G.

RESULTS

Synthesis and Structural Properties of HS Mimetics—A series of HS mimetics was synthesized, these being composed of cyclitol subunits, which represent pseudo-sugars in which the pyranosyl-ring oxygen has been replaced by a methylene unit. Such compounds are highly stable and "immune" from glycosidic cleavage. The synthesis and physicochemical properties of these compounds will be described in detail elsewhere.² Briefly, the synthesis involved the biotransformation of iodobenzene using the genetically engineered microorganism *Escherichia coli* JM109 (pDTG601), which overexpresses toluene dioxygenase, to yield the corresponding iodinated *cis*-1,2-dihydrocatechol as starting material (30, 31). A suitably protected epoxy derivative of this starting material was then reacted with the relevant 1, ω -diaminoalkane or -arene to give, after appropriate deprotection and de-iodination, the polyhydroxylated precursor to compounds of the general type 1 (Fig. 1). *cis*-Dihydroxylation of the double bond within the tetrameric cyclitols formed as

² M. G. Banwell, A. J. Edwards, V. Ferro, C. Freeman, L. Liu, and C. R. Parish, manuscript in preparation.

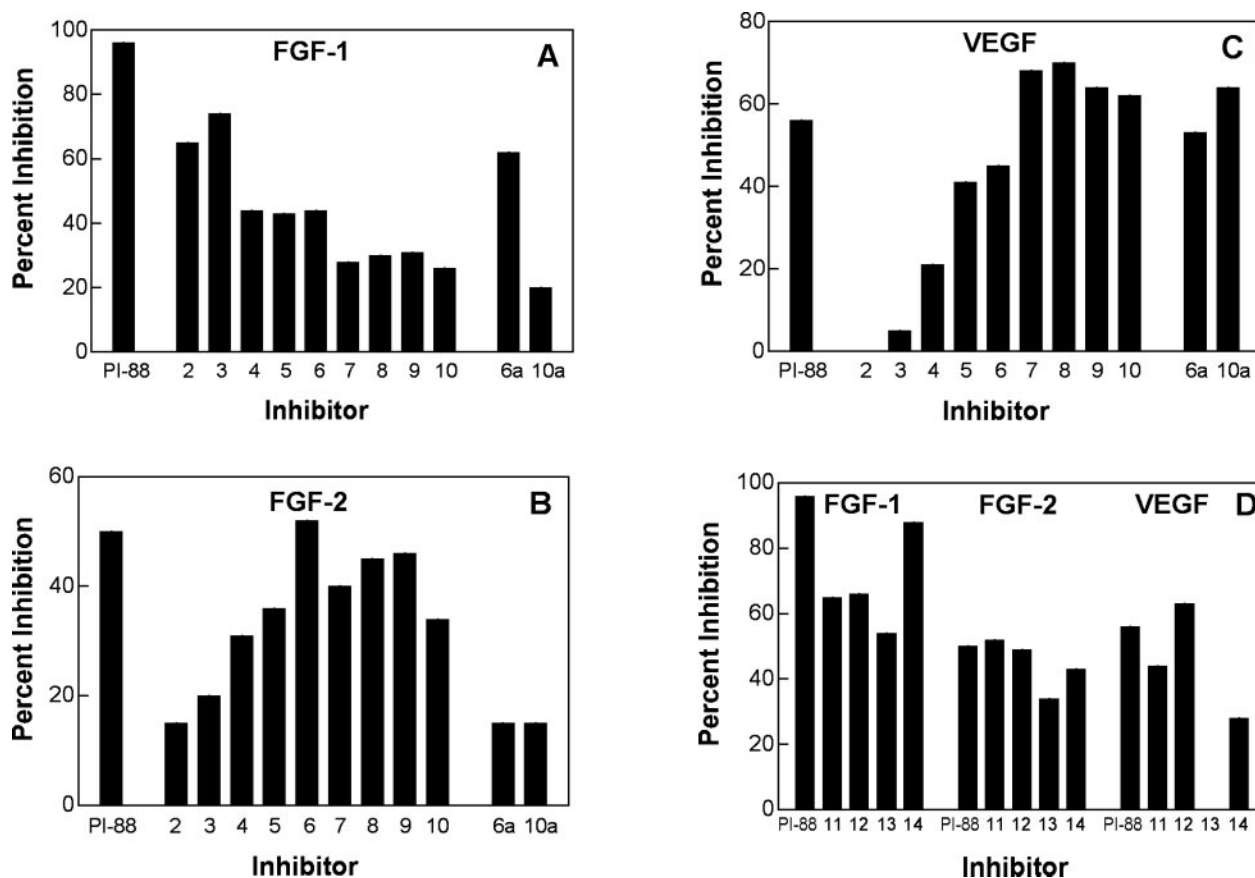


FIG. 2. Ability of a range of sulfated linked cyclitols to inhibit the binding of FGF-1, FGF-2, and VEGF to immobilized heparin. Data obtained using an optical biosensor and presented as percentage inhibition of growth factor binding relative to control binding in the absence of an inhibitor. In each case equimolar concentrations of growth factor and inhibitor were used, i.e. 25 nM for FGF-1, 34 nM for FGF-2, and 25 nM for VEGF. The heparan sulfate mimetic, PI-88, was included as a positive control. Inhibitory activity of the alkyl-linked cyclitols is depicted in panels A–C, and that of other linked cyclitols is depicted in panel D. Data values are the mean of at least two to three determinations with a variation of $\leq \pm 5\%$.

just described gave, after deprotection, the precursors to compounds of the form **1a** (Fig. 1). Exhaustive sulfation of each member of these two series of polyhydroxylated tetramers with pyridine-SO₃ followed by work up with sodium bicarbonate then afforded the target compounds **1** or **1a**, which represent two families of related compounds (Fig. 1). In one family, each pseudo-sugar unit contains three sulfation sites (**1**, Fig. 1), whereas with the other family five sulfation sites were incorporated within each unit (**1a**, Fig. 1). A less sulfated pentameric cyclitol was also generated by employing a triamino-linker (**14**, Fig. 1). In the case of the sulfated tetrameric cyclitols a range of linkers was used, namely alkyl chain linkers varying in length from 2 to 10 carbon atoms (**2–10**, **6a**, and **10a**, Fig. 1), *p*-xylyl and *m*-xylyl linkers (**11** and **12**, Fig. 1), and a sulfated propylene linker (**13**, Fig. 1). Compounds **6a** and **10a** represent more highly sulfated tetrameric cyclitols (**1a**) linked by 6-carbon and 10-carbon alkyl chains, respectively. Thus, a total of 15 cyclitol-based HS mimetics were employed in this study, the key structural features of these compounds being the presence of highly sulfated regions separated by spacers of variable length, flexibility, orientation, and hydrophobicity.

Ability of Heparin and PI-88 to Interact with Several Heparin/HS Binding Proteins—The binding properties of 10 heparin/HS-interacting proteins were investigated in this study (Table I). The proteins examined consisted of four heparin/HS binding growth factors (FGF-1, FGF-2, VEGF₁₆₅, and HGF), two chemokines (IL-8 and eotaxin), one HS-binding enzyme (LpL), and three heparin-inhibitable enzymes (heparanase, cathepsin G, and elastase). The ability of the different HS mimetics to inhibit the binding of the growth factors, chemokines, and LpL to heparin/HS was quantified using an optical biosensor with the biosensor chips carrying immobilized heparin or HS. In each case concentrations of the binding proteins were used, which exhibited ~60–90% maximal binding as detected by the biosensor. In the case of the heparin-inhibitable enzymes standard enzyme assays were established for each enzyme, and the ability of the different compounds to inhibit enzymatic activity was quantified.

In initial studies bovine lung heparin and the sulfated oligosaccharide, PI-88, were assessed for their inhibitory activity with IC₅₀ values being presented in Table I. The biosensor data presented in Table I are those obtained using immobilized heparin, but comparable results were observed when immobilized HS was used, although due to there usually being fewer protein binding sites on HS (relative to heparin) the sensorgram response was considerably lower with immobilized HS. The one exception to this rule was eotaxin, which interacted strongly with immobilized heparin but failed to bind to immobilized HS. PI-88 exhibited substantial inhibitory activity against seven of the heparin/HS-interacting proteins tested, namely FGF-1, FGF-2, VEGF₁₆₅, HGF, LpL, heparanase, and cathepsin G. On the other hand, PI-88 was essentially inactive against the two chemokines (IL-8 and eotaxin) and elastase.

Of the seven heparin/HS-binding proteins whose activity was blocked by PI-88, heparin was usually a more effective inhibitor than PI-88. Thus, in terms of IC₅₀, PI-88 was ~2 (FGF-1)-, 7 (FGF-2)-, 6 (HGF)-, 15 (LpL)-, 8 (heparanase)-, and 6 (cathepsin G)-fold less active than heparin (Table I). The one exception was VEGF₁₆₅ where PI-88 and heparin were equally effective. These differences are probably due to heparin being a polydisperse linear polysaccharide with a mean molecular mass of ~12.5 kDa and, therefore, potentially having multiple protein binding sites, whereas the much smaller PI-88 (~1.4–3.1 kDa) can probably only accommodate 1–2 heparin/HS-binding proteins per molecule. The fact that, in most cases, the IC₅₀ for heparin was substantially lower than the protein concen-

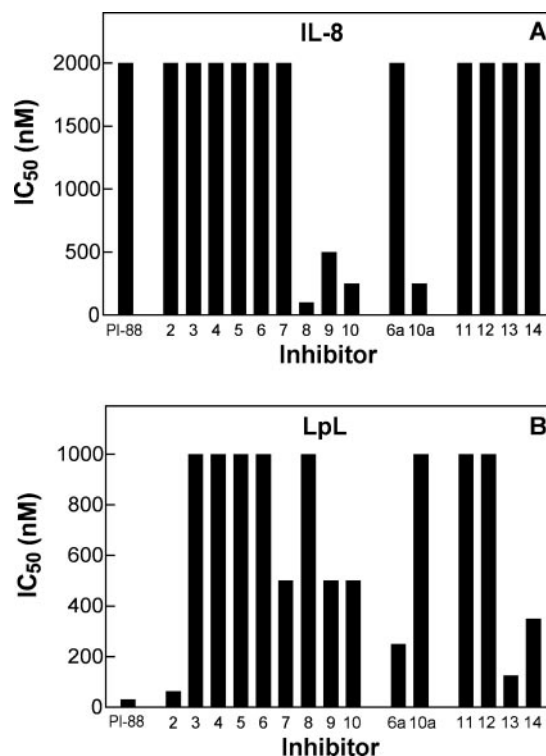


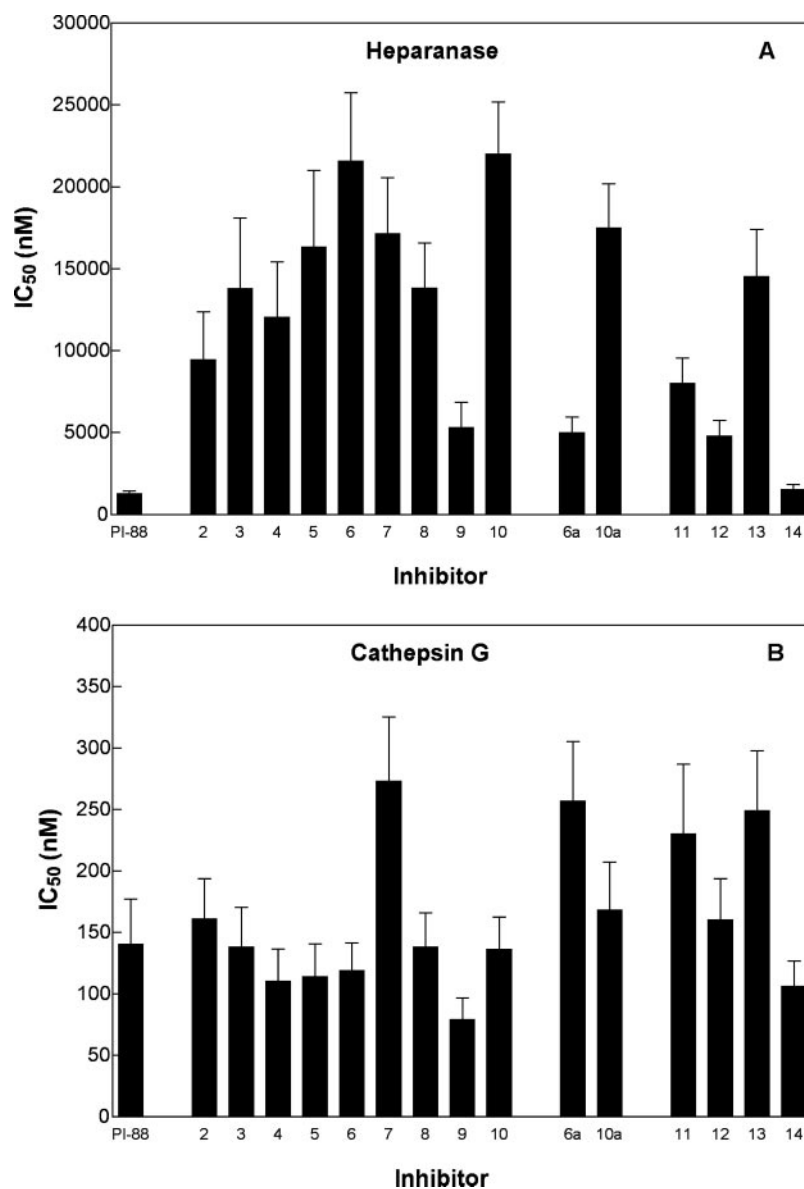
FIG. 3. Ability of a range of sulfated linked cyclitols to inhibit the binding of IL-8 and LpL to immobilized heparin. Data obtained using an optical biosensor and presented as concentration (nanomolar) of each inhibitor required to block binding of IL-8 (68 nM) (A) or LpL (50 nM) (B) by 50% (IC₅₀). The heparan sulfate mimetic, PI-88, is included for comparison. Data values are the mean of at least two to three determinations with a variation of <±5%.

tration used in the biosensor binding assays highlights this point, with LpL being an extreme example, *i.e.* 50 nM LpL was used in the binding assay but the heparin IC₅₀ was only 2 nM.

Ability of Sulfated Cyclitols to Block Protein-HS Interactions on the Optical Biosensor—The next series of experiments utilized the optical biosensor to assess the ability of the 15 above-mentioned sulfated cyclitols to inhibit the binding of the four growth factors (FGF-1, FGF-2, VEGF₁₆₅, and HGF), two chemokines (IL-8 and eotaxin), and LpL to immobilized heparin. When appropriate the different compounds were compared with PI-88 for their inhibitory activity. As with the data presented in Table I, comparable results were obtained with immobilized heparin and HS, but only the heparin data is presented here.

In the case of FGF-1 and FGF-2 binding to immobilized heparin, all 15 sulfated cyclitols exhibited some inhibitory activity. However, a comparison of the percentage inhibition of binding when equimolar concentrations of the inhibitor and growth factor were used is very informative (Fig. 2). In the case of the less sulfated tetrameric cyclitols containing alkyl chain spacers (compounds **2–10**), inhibition of FGF-1 binding was most effective and approaching that of PI-88 with the shorter (2–3 carbon atom) alkyl spacers, whereas the converse was true with FGF-2, compounds with alkyl spacers >5 carbon atoms being the most effective (Fig. 2, A and B). Interestingly, the more highly sulfated alkyl chain-linked tetrameric cyclitols (compounds **6a** and **10a**) were somewhat less effective at inhibiting FGF-2 binding than their less sulfated counterparts (compounds **6** and **10**). Alkyl chain length was even more critical in determining the inhibitory activity of the sulfated tetra-cyclitols for the VEGF₁₆₅-heparin interaction (Fig. 2C). Compound **2** was essentially inactive, with increasing alkyl chain length from 3–7 carbon atoms steadily increasing inhibitory

FIG. 4. Ability of a range of sulfated linked cyclitols to inhibit the enzymatic activity of heparanase and cathepsin G. Enzyme assays performed as described under "Experimental Procedures" with data being presented as concentration (nanomolar) of each inhibitor required to reduce enzymatic activity of heparanase (A) or cathepsin G (B) by 50% (IC_{50}). The heparan sulfate mimetic, PI-88, is included for comparison. IC_{50} values derived from pooled data from 8–10 determinations with vertical bars representing S.E.



activity to the level of PI-88. Also, unlike FGF-2, increasing the extent of sulfation of the cyclitol units did not change inhibitory activity, *i.e.* compare compounds **6a** and **10a** with **6** and **10**. Sulfated tetracyclitols containing a *p*-xylyl or *m*-xylyl linker (compounds **11** and **12**) or the sulfated pentameric cyclitol (compound **14**) were essentially as effective as PI-88 at blocking FGF-1, FGF-2, and VEGF₁₆₅ binding (Fig. 1D). However, compound **13**, a sulfated tetracyclitol linked by a sulfated propylene moiety, was unable to block VEGF₁₆₅ binding despite being quite an effective inhibitor of the interaction of FGF-1 and FGF-2 with heparin/HS (Fig. 1D). Thus, incorporating a sulfate group in the 3-carbon atom alkyl spacer did not result in inhibitory activity against VEGF₁₆₅, *i.e.* both compounds **3** and **13** had little or no VEGF-specific inhibitory activity. Finally, unlike the other heparin/HS-binding growth factors, the binding of HGF (20 nM) to immobilized heparin was not significantly affected by any of the 15 sulfated cyclitols tested. Thus, even when using sulfated cyclitol concentrations as high as 0.5–1.0 μ M negligible inhibitory activity was observed (data not shown), despite PI-88 having an IC_{50} for HGF of 30 nM (Table I).

In contrast with FGF-1, FGF-2, and VEGF, a totally different pattern of inhibition was obtained with the chemokines

eotaxin and IL-8. As discussed earlier, PI-88 was unable to block the binding of both chemokines to immobilized heparin (Table I), and all 15 sulfated cyclitols were also inactive against eotaxin (data not shown). On the other hand, 4 of the 15 sulfated cyclitols did effectively block the IL-8-heparin interaction, with the remaining 11 compounds being totally ineffectual (Fig. 3A). Thus, alkyl-linked tetrameric cyclitols were inactive unless a spacer of >7 carbon atoms was used, an extension of the alkyl chain spacer from 7 to 8 carbon atoms resulting in at least a 40-fold increase in inhibitory activity. In fact, compound **8** displayed an IC_{50} (50 nM) essentially equimolar with the IL-8 concentration (68 nM) used in the binding assay. Compounds **9** and **10** were also quite strong inhibitors, although not as effective as compound **8**, with the more highly sulfated **10a** giving comparable inhibition to **10**.

Yet another unique inhibition profile was obtained with LpL (Fig. 3B). In this case compound **2** was the most active, a lengthening of the alkyl chain spacer from 2 to 3 carbon atoms resulting in a >20-fold reduction in inhibitory activity. Some of the sulfated tetrameric cyclitols with longer alkyl chain spacers (compounds **7**, **9**, and **10**) also showed weak inhibitory activity. The more highly sulfated **6a** was also a modest inhibitor unlike the structurally similar but less sulfated **6**. Similarly, the sul-

fated pentameric cyclitol (compound **14**) was a relatively weak inhibitor. In fact, compound **13** was the only other cyclitol with an IC_{50} approaching that of compound **2**. This result is particularly interesting, because it implies that the insertion of a centrally placed, negatively charged, sulfate group in the propylene chain spacer converts this construct from an inactive to an active compound, *i.e.* compare the IC_{50} of **13** with that of **3**.

Inhibition of ECM-degrading Enzymes by Sulfated Cyclitols—The different sulfated cyclitols were assessed for their ability to inhibit the enzymatic activity of a number of ECM-degrading enzymes. PI-88 was originally developed as a heparanase inhibitor, so it was of considerable interest to determine the heparanase inhibitory activity of the 15 sulfated cyclitols (Fig. 4A). It was found that only one of the synthetic molecules, sulfated pentameric cyclitol (**14**), was as active as PI-88, presumably because this entity presents a relatively linear array of sulfated pseudo-sugars to the active site of the enzyme. Although the other sulfated cyclitols were relatively poor heparanase inhibitors, some structure-activity relationship information could be gleaned from the data. For example, relative to alkyl chain length, IC_{50} values followed a bell-shaped curve with the 2- and 9-carbon spacers producing the most active compounds and the sulfated tetracyclitol containing a 6-carbon spacer (**6**) being virtually inactive. It is noteworthy, however, that the more highly sulfated version of compound **6** (*i.e.* **6a**) possessed similar activity to compound **9**. In direct contrast to the situation observed with LpL, insertion of a sulfate group in the propylene spacer had no effect on inhibitory activity. Interestingly, increasing the alkyl chain length from 9 to 10 carbon atoms abolished inhibitory activity with the more highly sulfated **10a** also being a poor heparanase inhibitor. Finally, sulfated tetrameric cyclitols containing the more rigid xylyl linkers were modest heparanase inhibitors.

In the case of the proteases elastase and cathepsin, as with PI-88 (Table I), none of the 15 cyclitols proved capable of inhibiting the enzymatic activity of elastase (data not shown). In contrast, all of the sulfated cyclitols effectively inhibited cathepsin G with most IC_{50} values only varying over a 2- to 3-fold range and resembling that of PI-88 (Fig. 4B). These data suggest that the heparin/HS binding site of cathepsin G is highly promiscuous and binds to short, highly negatively charged, sugar sequences. One interesting feature of the cathepsin G inhibition data, however, was that some of the compounds only partially inhibited total enzymic activity. For example, at high concentrations heparin and PI-88 reduced cathepsin G activity by 90–95%, whereas compounds **6** and **10** only reduced the total activity of the enzyme by ~60 and ~80%, respectively (Fig. 5A), despite PI-88, **6**, and **10** having comparable IC_{50} values (Fig. 4B). A similar situation was seen in the LpL binding-inhibition experiments. Compounds **2** and **13** were the most active sulfated cyclitols in this system with the former resembling PI-88 in terms of IC_{50} value (Fig. 3B). However, compound **2** consistently only blocked about 75% of LpL binding compared with 90% by PI-88 (Fig. 5B). This effect was even more extreme with compound **13**, which only blocked LpL binding by ~60% at the highest concentration tested despite PI-88, **2**, and **13** blocking LpL binding to comparable levels when used at very low concentrations (Fig. 5B).

DISCUSSION

Defining the structural motifs in HS that interact with different heparin/HS-binding proteins is a major challenge. The usual approach has been to isolate HS fragments that bind to particular proteins and then determine the structure of the reactive fragments (32, 33). This is a difficult procedure as there are often multiple binding motifs in HS of low abundance and thus obtaining detailed structural information, particu-

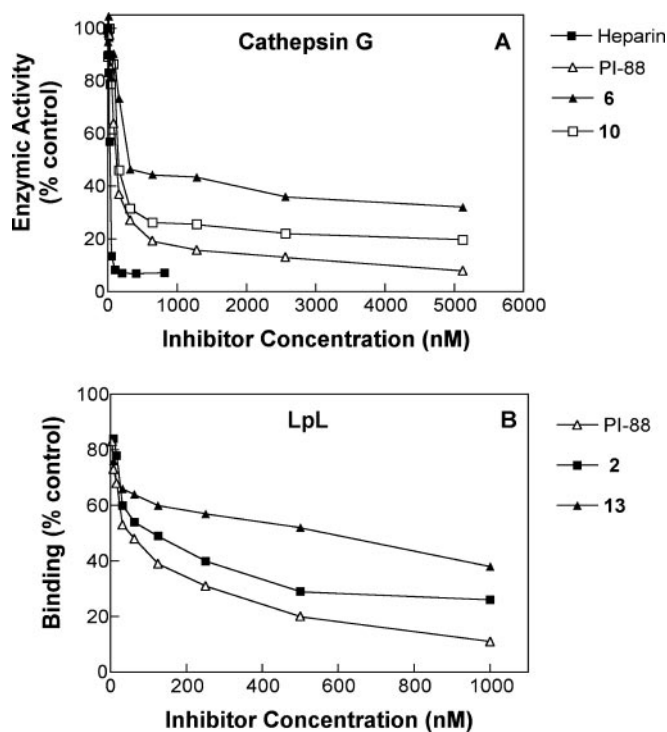


FIG. 5. Partial inhibition of cathepsin G enzymatic activity and LpL binding to immobilized heparin by certain sulfated linked cyclitols. A, cathepsin G data showing that compounds **6** and **10** only partially inhibit cathepsin G enzymatic activity, with heparin and the heparan sulfate mimetic, PI-88, being included as controls. B, LpL binding to immobilized heparin as measured by an optical biosensor, with compounds **2** and **13** exhibiting partial inhibition of LpL binding. The heparan sulfate mimetic, PI-88, is included for comparison. Data from the single experiment shown are representative of three experiments conducted.

larly of lengthy HS fragments, is a complex process. In an attempt to overcome these difficulties we examined the ability of a small family of well defined HS mimetics to interact with 10 functionally diverse heparin and/or HS-binding proteins. A major advantage of this approach is that it is possible to rapidly identify differences in binding specificity between different HS-binding proteins, with a specific inhibitor of a particular protein-heparin/HS interaction being immediately available as a potential therapeutic agent.

The HS mimetics used in this study consisted of cyclitol-based pseudo-disaccharides that were “dimerized” using a range of linker molecules. Alkyl chain linkers provided highly hydrophobic and flexible spacers of variable chain length. In contrast, the *p*-xylyl and *m*-xylyl linkers represent rigid and non-polar spacers with varied orientations, whereas the addition of another cyclitol unit or a sulfated propylene chain as a linker resulted in structures containing sulfated spacers. The extent of sulfation of the individual cyclitol units was also varied from three to five sulfate groups per unit. This level of sulfation is much higher than would normally occur in heparin or HS, but the potential exists in the future to synthesize sulfated cyclitols with more limited and highly regio- and/or stereo-specific sulfation patterns. Despite the high level of sulfation, 7 of the 10 proteins examined displayed a unique reactivity pattern with the panel of sulfated cyclitols. The exceptions were HGF, eotaxin, and elastase, with none of the 15 sulfated cyclitols tested blocking the interaction of HGF and eotaxin with heparin/HS or inhibiting elastase enzymatic activity. In the case of eotaxin and elastase this result was not surprising, because the sulfated oligosaccharide, PI-88, also failed to interact with these proteins.

Each of the HS-binding growth factors tested displayed characteristic inhibition patterns with the most informative compounds being the alkyl chain-linked tetrameric cyclitols. Thus, in terms of blocking heparin/HS binding, compounds with a spacer of 2–3 carbon atoms were the most inhibitory for FGF-1 but were the least inhibitory for FGF-2 and VEGF₁₆₅, compounds with the longer alkyl chain linkers being most effective against the latter two growth factors. Despite these differences many of the sulfated cyclitols exhibited substantial inhibitory activity against the three growth factors, particularly FGF-1 and FGF-2. This finding is consistent with earlier studies that suggest that, although FGF-1 and FGF-2 bind HS sequences that vary in the position and number of 6-*O*-sulfates, they bind a range of HS sequences and there is substantial overlap in the sequences they recognize (3, 18). In contrast, sulfated cyclitols with short linkers (*e.g.* compounds **2**, **3**, and **13**) were essentially inactive against VEGF₁₆₅ suggesting that this growth factor interacts with long stretches of negatively charged residues in HS. Earlier studies actually support this interpretation, because VEGF₁₆₅, the most abundant isoform of VEGF, is most effectively inhibited from binding to VEGF receptors by lengthy heparin fragments composed of 16–18 sugar units (34). On the other hand, the failure of any of the sulfated cyclitols to block the HGF-heparin interaction is surprising, because HGF is known to bind to relatively short HS sequences (35). Perhaps HGF is the one example in this study where the highly sulfated nature of the cyclitol derivatives interfered with their ability to interact with the growth factor.

Much more selective inhibition patterns were observed with IL-8 and LpL. IL-8, a member of the CXC-chemokine family, exists as a homodimer, and it has been suggested that the molecule interacts with HS fragments containing two highly sulfated hexasaccharides joined by a non- or under-sulfated dodecasaccharide linker (36). The results obtained with the sulfated cyclitols support this interpretation, because inhibitory activity was highly dependent on the chain length of the alkyl linkers, *i.e.* a linker of >7 carbon atoms was essential. Presumably, linker length is crucial to allow the linked sulfated pseudo-disaccharides to interact with the HS-binding site of each subunit of the IL-8 dimer. In contrast, none of the sulfated compounds tested, including PI-88, could block eotaxin binding to heparin. Eotaxin also exists as a homodimer (37), so it is likely that the appropriately spaced sulfated motifs were not present in the limited family of sulfated molecules tested. Sulfated cyclitol linker length was also critical for LpL binding inhibition, although in this case a very short spacer was required for optimal inhibitory activity. Enzymatically active LpL exists as a homodimer that has a high affinity for heparin and HS and interacts most strongly with heparin octa- to decasaccharides (38). Our results suggest that the spatial arrangement of the sulfated regions in HS are critical, perhaps to adequately bridge the LpL dimers, as is the case with the chemokines.

Finally, certain sulfated cyclitols were also found to be potent enzyme inhibitors, in some cases achieving the potency of PI-88. The general conclusion from the heparanase inhibition studies was that HS mimetics that resemble a sulfated pentasaccharide were the most effective inhibitors. Compounds that contained non-sulfated hydrophobic linkers were poor inhibitors. In contrast, all of the sulfated cyclitols were quite potent cathepsin G inhibitors, suggesting that this enzyme reacts with a wide range of short polyanions. One intriguing feature of the cathepsin G data, however, was the observation that only a subpopulation of enzyme molecules were inacti-

vated by the sulfated cyclitols, the extent of partial inhibition varying from compound to compound. Similar partial inhibition of heparin binding was also observed with LpL and compound **13**. These data suggest that both cathepsin G and LpL exist in states that exhibit differential HS-binding properties. The functional relevance of this phenomenon may be that it prevents HS fragments released from the ECM at sites of inflammation and tissue repair from totally displacing LpL from the matrix or totally inactivating ECM-degrading enzymes such as cathepsin G.

Collectively this study has highlighted the power of using a simple panel of HS mimetics to delineate differences in the HS binding specificity of proteins. Clearly the spatial separation of anionic groups within the HS mimetics plays a critical role in determining the specificity of interaction. This point is most clearly evident with two of the sulfated tetrameric cyclitols, compounds **2** and **8**, which only differ by the length of their alkyl chain spacer, *i.e.* 2 and 8 carbon atoms, respectively. Despite this similarity in structure compound **2** is a modest inhibitor of FGF-2 and a very effective inhibitor of FGF-1 and LpL binding to heparin/HS but is inactive against VEGF and IL-8, whereas compound **8** possesses exactly the opposite properties, being a modest inhibitor of FGF-1 binding, and a strong inhibitor of FGF-2, VEGF, and IL-8 binding. Such data also indicate that an HS mimetic approach provides a means of rapidly identifying compounds that may have therapeutic value by specifically blocking the action of certain HS-binding proteins in pathological situations.

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Use of Sulfated Linked Cyclitols as Heparan Sulfate Mimetics to Probe the Heparin/Heparan Sulfate Binding Specificity of Proteins
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